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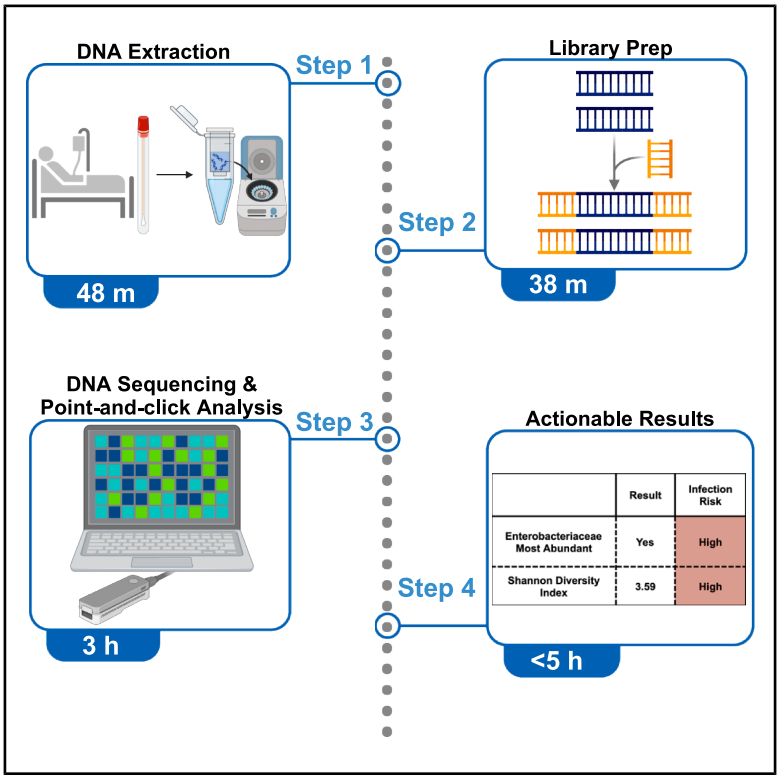
ON-Time enables rapid microbiome sequencing and analysis for precision medicine

Graphical abstract



Authors

Colin MacKenzie, Heewon Seo, Jared Schlechte, ..., Kathy D. McCoy, Christina S. Thornton, Braedon McDonald

Correspondence

bamcdona@ucalgary.ca

In brief

MacKenzie et al. present ON-Time, a rapid metagenomic sequencing and analysis workflow that characterizes microbial composition of individual patient fecal samples in <5 h. Validated against mock communities and shotgun sequencing, ON-Time offers a cost-effective approach for identifying clinically relevant microbiome features that may help inform personalized, microbiome-guided care.

Highlights

- We present ON-Time, a method to analyze microbiomes of patient fecal samples
- ON-Time enables rapid turnaround from patient sample to actionable microbiome results
- ON-Time is cost effective for on-demand individual patient sample analysis

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ON-Time enables rapid microbiome sequencing and analysis for precision medicine

Colin MacKenzie,[1,2] Heewon Seo,[2] Jared Schlechte,[1,2] Aydin Herik,[2,3] Ish Bains,[1] Ian-Ling Yu,[1,2] Kathy D. McCoy,[2,4] Christina S. Thornton,[2,5,6] and Braedon McDonald[1,2,7,*]

[1]Department of Critical Care Medicine, Cumming School of Medicine, University of Calgary, Hospital Dr NW, Calgary, AB 3330, Canada
[2]Snyder Institute for Chronic Diseases, Cumming School of Medicine, University of Calgary, Hospital Dr NW, Calgary, AB 3330, Canada
[3]Undergraduate Medical Education, Cumming School of Medicine, University of Calgary, Hospital Dr NW, Calgary, AB 3330, Canada
[4]Department of Physiology and Pharmacology, Cumming School of Medicine, University of Calgary, Hospital Dr NW, Calgary, AB 3330, Canada
[5]Department of Microbiology, Immunology, and Infectious Diseases, Cumming School of Medicine, University of Calgary, Hospital Dr NW, Calgary, AB 3330, Canada
[6]Department of Medicine, Cumming School of Medicine, University of Calgary, Hospital Dr NW, Calgary, AB 3330, Canada
[7]Lead contact
*Correspondence: bamcdona@ucalgary.ca
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MOTIVATION Conventional microbiome sequencing for use in precision medicine is limited by long turnaround times, complex procedures, and high costs. We aimed to address these limitations by developing simplified and rapid wet-lab workflows coupled with point-and-click data analysis to enable rapid, accurate, and cost-effective microbiome analysis.

SUMMARY

Clinical application of microbiome-guided therapies in the intensive care unit (ICU) requires a method to rapidly analyze patient microbiomes to guide urgent treatment decisions. Conventional microbiome sequencing and analysis methods require long turnaround times, methodological complexity, and high costs that are barriers to clinical application. Here, we describe a method for rapid (<5 h turnaround from sample to results) and accurate metagenomic sequencing and taxonomic analysis of microbiomes in individual patient fecal samples, called ON-Time. ON-Time uses a simplified and rapid wet-lab workflow coupled with point-and-click data analysis. Accuracy and precision of ON-Time data were validated using defined mock microbial communities and head-to-head comparison with conventional shotgun metagenomics of ICU patient samples. Key limitations include stochastic identification of functional genes such as antimicrobial resistance and virulence factors. Taken together, ON-Time offers a rapid, accurate, and cost-effective method to analyze individual patient samples for clinically actionable microbiome features to guide personalized therapeutics.

INTRODUCTION

Human and animal research has established that pathological alterations to the gut microbiome contribute to pathogenesis and adverse outcomes of many acute medical diseases, leading to the development of microbiome-modifying therapies that are poised for clinical trial testing and real-world application.[1–9] However, there remains a crucial barrier to actioning precision microbiome-guided therapies—conventional methods to analyze the microbiome of patient samples require excessively long turnaround times, methodological complexity, and high costs that are impractical to guide individual patient care in the acute medical setting where decision-making must occur as fast as possible.[10] This is particularly true in the intensive care unit (ICU), where time-to-intervention is a key determinant of clinical outcomes.[11] Therefore, actioning microbiome-guided therapies in the real-world setting of the ICU requires the development of a rapid, accurate, and cost-effective method to analyze individual patient's gut microbiome for targeted (personalized) therapy. To address this need in the field of translational microbiome science,[10] we undertook a study to develop and validate a method for rapid and clinically feasible microbiome analysis of individual patient samples, called ON-Time.

Contemporary metagenomic methods for microbiome analysis primary rely on sequencing of PCR-amplified microbial marker genes (16S rRNA amplicon sequencing) or short-read shotgun

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sequencing.[12] However, these conventional sequencing methodologies present critical limitations for application in acute medical care. 16S rRNA amplicon sequencing is limited solely to bacterial profiling and restricted taxonomic resolution (typically genus level at best).[12] Furthermore, current sequencing platforms are optimized for multiplexing hundreds of samples to enable cost-effectiveness and are therefore not feasible for on-demand individual patient sample analysis. Lastly, the process from patient sample to data (DNA extraction, marker gene PCR amplification, barcoding, cleanup, sequencing, and analysis) can take days to weeks to complete, which yields a turnaround time that is incompatible with acute clinical decision-making in the ICU. Shotgun metagenomics provides comprehensive multi-kingdom and functional genomic analyses of the microbiome but presents even greater cost and turnaround-time limitations to its clinical application. Collectively, these sequencing technologies, which represent the current "gold-standard" in the laboratory research realm, are not feasible for the acute medical setting, and thus there is a crucial need for a rapid, comprehensive, cost-effective method to analyze the microbiome of individual patient samples, on demand, to facilitate microbiome-guided care in the clinical setting of the ICU and other acute medical environments.

To fill this gap, we developed, optimized, and validated a method called "ON-Time" that enables rapid, cost-effective, user-friendly analysis of the gut microbiome in individual critically ill ICU patients. Starting with rectal swabs collected from ICU patients, ON-Time provides robust ecological and taxonomic data of the bacterial and fungal microbiome in less than 5 h. The accuracy and precision of ON-Time were validated using defined mock community samples and paired comparison of patient samples to "gold-standard" short-read shotgun metagenomic sequencing. Collectively, this study reveals that ON-Time provides a feasible method for on-demand, individual patient microbiome analysis to facilitate personalized and targeted microbiome-guided care in the clinical setting.

RESULTS

ON-Time rapid microbiome analysis to characterize the fecal microbiome of individual critically ill patients in the ICU

We aimed to develop an optimized workflow for rapid microbiome analysis of fecal samples from ICU patients that includes DNA isolation, sequencing library preparation, DNA sequencing, and bioinformatic analysis of clinically actionable microbiome metrics, which we call ON-Time (Figures 1A and 1B). Comparing common commercially available sequencing platforms, we identified that Oxford Nanopore Technologies (ONT) MinION-based workflows using Flongle flow cells were optimal for our objectives. First, ONT-based long-read sequencing allows for real-time sequencing data output and analysis (enabling rapidity of analysis), flexibility to run individual samples or multiple (multiplexed) samples on the same flowcell (crucial for clinical feasibility), as well as the most cost-effective platform for single-patient sample on-demand analysis (Table S1). Specifically, when running a single sample using ON-Time, the Flongle flow cell cost is ~$110 CAD, which is ~$535 CAD less than the next most affordable rapid conventional sequencing flow cell if amortized for an individual patient sample (Table S1). Thus, to achieve the feasibility goals of rapidity, cost-effectiveness, and user-friendliness, we elected to develop our rapid microbiome analysis pipeline using the ONT MinION Mk1B sequencing device using Flongle flow cells, supported by the Flongle adapter. Importantly, with this approach, sequencing and bioinformatic analysis are contained within a single computer workstation connected to the ONT sequencing device. Thus, this pipeline can be accomplished with a single laptop computer connected to a Mk1B device that is smaller than a mobile phone, which is beneficial for both efficiency of the workflow and security of the patient data.

Fecal samples were collected from participants admitted to the ICU using rectal swabs, which is crucial to enable on-demand collection that is independent of the timing of bowel motions (which are highly unpredictable in critically ill individuals). Rectal swabs were then immediately processed for DNA extraction using the Qiagen DNeasy PowerSoil Kit (Figure 1C). Next, isolated DNA underwent library preparation using the Rapid Sequencing Kit V14 from Oxford Nanopore Technologies, which is a rapid PCR-free, tagmentation-based barcoded library prep (Figure 1D). To confirm that these wet lab procedures could be completed within a feasible timeline, we measured the time required to complete each step of this simplified wet lab workflow over 31 sample iterations and observed a rapid learning curve culminating in a total duration of ~1.75 h from rectal swabs to fully prepared sequencing libraries (Figures 1B–1D).

To define the optimal duration of DNA sequencing to yield accurate and precise microbiome data from patient samples, the prepared DNA libraries were loaded into sequencing flowcells (ONT Flongle), sequencing was initiated, and total FASTQ files were exported every 30 min for a total of 20 h. Following export of the raw sequencing data, taxonomy was assigned using Kraken2 within the Epi2Me software (ONT). First, we quantified the total number of reads accumulating over time, which demonstrated rapid accumulation of reads over the first 8 h of sequencing, approaching a plateau by 20 h (Figure 1E). Of note, median read length for each sample was stable over the course of sequencing (Figure 1E). We quantified the total number of assigned taxa at the species level over the course of sequencing and similarly found exponential accumulation of unique species over the first ~10 h of sequencing, which progressively plateaued by 20 h (Figure 1F).

Next, we focused our analysis on two key microbiome metrics that have been identified in prior literature of critically ill patients as associated with adverse outcomes and thus potentially clinically actionable in patient management[3]—the Shannon Diversity index (low Shannon diversity has been associated with adverse outcomes in ICU patients) and the relative abundance of gut pathobionts (expansion of *Enterobacteriaceae* and *Enterococcaceae* taxa has been associated with adverse outcomes).[13–15] Of note, although these microbiome metrics are the focus of our current analysis based on existing literature in ICU patients, ON-Time is not limited to data on these metrics, as it can provide comprehensive taxonomic and functional genomic readouts (see further below). Real-time analysis of α-diversity by Shannon Diversity index (which describes within-sample taxonomic richness and evenness) revealed very rapid achievement of stable

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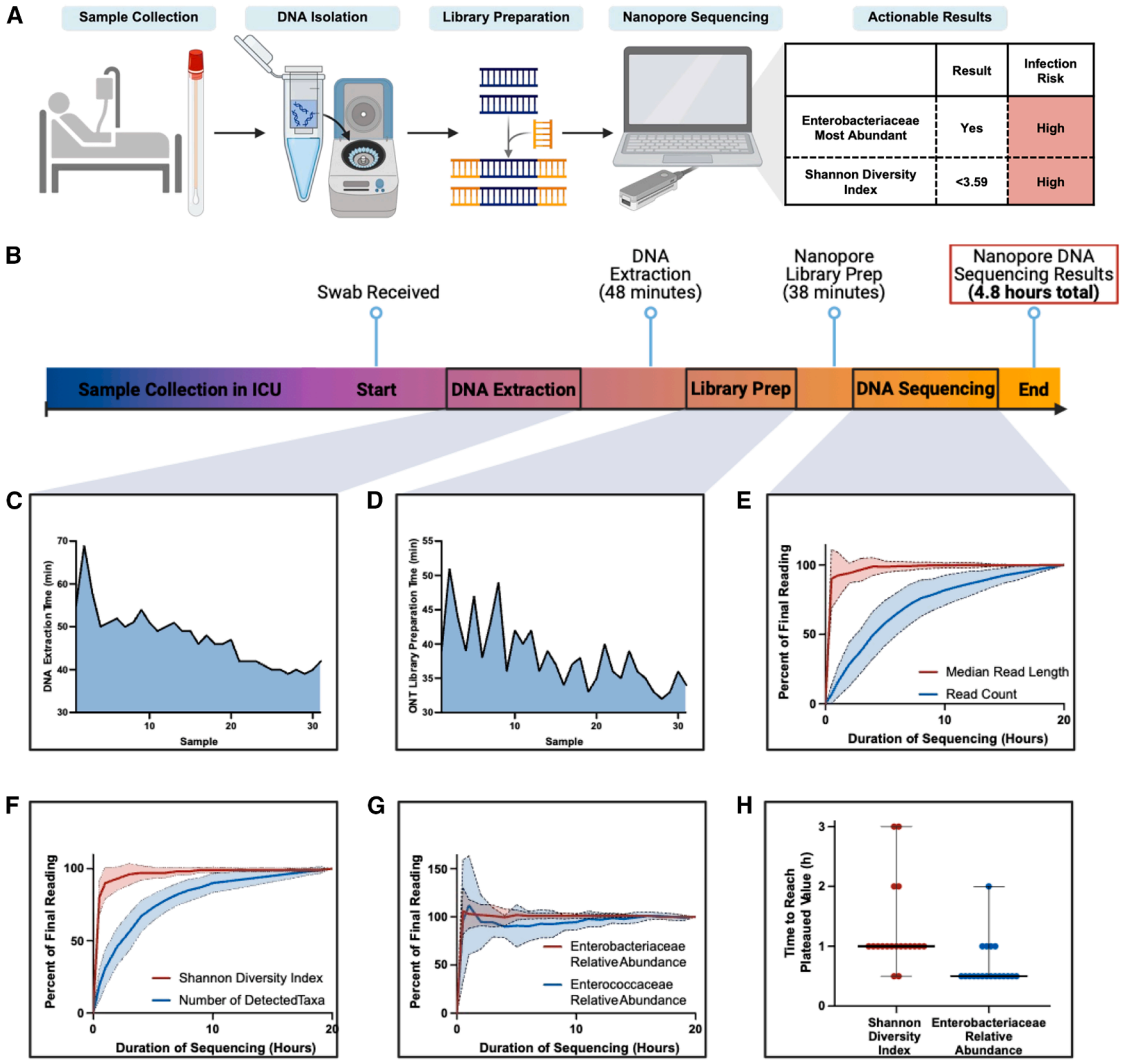

Figure 1. Development and optimization of ON-Time rapid microbiome analysis of fecal samples from critically ill patients

(A) Schematic overview of ON-Time rapid microbiome analysis process (illustration created in BioRender).

(B) Timeline for each step in ON-Time rapid microbiome sequencing process. Times represent median durations across 31 patient samples run through ON-Time (illustration created in BioRender).

(C) The amount of time required to complete DNA extraction from ICU patient rectal swab samples across 31 replicates of the ON-Time pipeline ($n = 31$ samples).

(D) The amount of time required to complete DNA sequencing library preparation from ICU patient rectal swab samples across 31 replicates of the ON-Time pipeline ($n = 31$ samples).

(E–G) After the initiation of ONT sequencing, total FASTQ files were analyzed every 30 min for a total of 20 h (final), and at each time point, we determined (E) the median read length and total read counts, (F) total number of detected taxa and calculated Shannon Diversity Index, and (G) relative abundance of *Enterobacteriaceae* and *Enterococcaceae* taxa. Data are reported as percentage of final reading (i.e., reading at 20 h of sequencing) at each data time point; line shows mean or median, and shaded area shows standard deviation ($n = 19$ individual patient samples).

(H) Plot shows the median duration of sequencing time required to reach plateau values for Shannon Diversity Index and *Enterobacteriaceae* relative abundance, rounded to the nearest 30 min [plateau defined as upper inflection point on curves in (F) and (G)]. Dots represent individual patient samples ($n = 19$), line shows median, and whiskers show range.

values, with a median time-to-final result across all samples of 1 h (range 0.5–3h) (Figure 1F). Similarly, real-time analysis of pathobiont relative abundance demonstrated *Enterobacteriaceae* relative abundance had a median time-to-final result of 0.5 h of sequencing (range 0.5–2 h), and *Enterococcaceae* relative abundance had a median time-to-final result of 0.5 h of sequencing (range 0.5–2 h) (Figure 1G). Thus, the maximum duration required for any sample to achieve stable final values of Shannon diversity or relative abundance of the specific taxa was 3 h of sequencing (2/19 samples required 3 h of sequencing to achieve final Shannon Diversity index value) (Figures 1H and S1). With this, we conclude that stable and final microbiome metrics of Shannon Diversity index, *Enterobacteriaceae* relative abundance, and *Enterococcaceae* relative abundance are

confidently achieved within 3 h of sequencing time (Figures 1H and S1). However, it is noteworthy that the collection of real-time sequencing data allows the user to see early trends in the data even before the maximum values are reached, as early as the first 30-min FASTQ output (Figures 1E–1H). Lastly, clinical users without advanced computational skills may want these microbiome data outputs translated into a clinically actionable summary while maintaining a point-and-click workflow from start to finish. Therefore, we developed a web-based app (https://apps.thebiohub.ca/ONTime/) into which the user can drag-and-drop output files from ONT sequencing real-time analysis (taxonomy profiles from Epi2Me software) that translates the raw taxonomy data into clinically actionable result (in this case, whether an ICU patient's microbiome Shannon-diversity and *Enterobacteriaceae* relative abundance predict elevated risk of infection[3]) (Figure 1A). Taken together, these data demonstrate that ON-Time rapid microbiome analysis achieves clinically actionable microbiome data from patient samples within ~4.8 h from sample collection (~1.75 h wet-lab time, ~3 h sequencing time, and real-time analysis; Figure 1B).

Multi-kingdom taxonomic validation and optimization of ON-Time using standardized microbial communities

To validate the accuracy and precision of ON-Time rapid microbiome analysis, we utilized two commercially available synthetic mock communities of microbes consisting of known composition and ratios of bacteria and fungi. The ZymoBIOMICS D6300 standard consists of eight bacterial taxa in even proportions as well as two fungal taxa in even proportions. The ZymoBIOMICS D6331 standard is a more complex community of 21 different bacterial, archaeal, and fungal strains found in the human gut microbiome, prepared in known proportions. For each mock community, we performed six iterations (n = 6 experimental replicates) of DNA extraction, library preparation, and sequencing using the ON-Time protocol described above. Individual runs produced expected variability, with between-run variability (9.3%–18.1% of explained variance) proving minimal compared to the between-community variability (75.7%–76.1% of explained variance) (Figures 2A and 2B). Further analysis of the bacterial community composition of ZymoBIOMICS D6331 versus D6300 mock communities confirmed the ability to resolve distinct clustering of the communities when analyzed at a species level (Figures 2A and 2B). Bacterial taxonomic analysis verified the ability to identify expected taxa within mock communities, with consistent accuracy (observed-to-theoretical abundance) (Figures 2C and 2D). Furthermore, consistent with our observations from clinical samples above, relative abundance of individual bacterial taxa within the more complex ZymoBIOMICS D6331 community rapidly stabilized within a median time-to-final result of 1 h (Figure 2C).

In addition to bacterial characterization, we evaluated fungal taxa within mock communities by ON-Time. Similar to bacterial taxa analyses, we observed that relative abundance values of *Candida albicans* within the ZymoBIOMICS D6331 community demonstrated a median time-to-final result of 2 h (range 2–3 h) (Figure 2E). Furthermore, evaluation of the accuracy of fungal identification after 3 h of ON-Time sequencing using observed-to-theoretical ratio of *C. albicans* and *Saccharomyces cerevisiae* within the ZymoBIOMICS D6331 community revealed median ratios of 101.5% and 92.45%, respectively (with 100% representing perfect accuracy; Figure 2F). Collectively, these mock community experiments validate the accuracy and precision of bacterial and fungal microbiome analysis using the ON-Time pipeline.

Comparative assessment of ON-Time rapid microbiome analysis to conventional shotgun metagenomics

We next aimed to ensure that data generated by ON-Time yield comparable data to the established "gold-standard" method for microbiome analysis—short-read shotgun metagenomics. We performed a head-to-head comparison of ON-Time microbiome analysis and short-read shotgun sequencing by Illumina NovaSeq 6000 of rectal swab samples collected from ICU patients (n = 29) and healthy volunteers (n = 5). Wet-lab workflows for library preparation are inherently different between ON-Time (rapid PCR-free tagmentation based barcoding library prep and sequencing on ONT flow cells) and short-read shotgun sequencing (PCR-free NEBNext Ultra II module, run on Illumina NovaSeq 6000). Library preparation for short-read shotgun sequencing requires significantly longer amount of time to prepare DNA libraries for Illumina-based sequencing compared to ON-Time (Table S1).

To enable harmonized evaluation of sequencing output from each method, we compared matched patient samples and utilized identical processing and data analysis pipelines for FASTQ files generated by both workflows. Head-to-head comparison of matched samples revealed no significant differences in microbiome community composition demonstrated by indistinguishable β-diversity between sequencing methods (Bray-Curtis dissimilarity distances, PERMANOVA p value = 0.2492; Figure 3A). Analysis of bacterial taxonomy within matched samples were also concordant at the family level (Figure 3B) and species level (Figure S2). Concordance was determined through Procrustes analysis of Bray-Curtis dissimilarity at both the family level (Procrustes r = 0.94, PROTEST p = 0.0001) and the species level (Procrustes r = 0.96, PROTEST p = 0.0001) for matched samples between conventional and ON-Time sequencing methods. Focusing further on clinically relevant metrics such as *Enterobacteriaceae* relative abundance, both sequencing methods were able to resolve differences in *Enterobacteriaceae* relative abundance between healthy volunteer samples and ICU patient samples (Figure 3C). Focusing specifically on the single most abundant family in each sample, we found an overall 88.2% agreement between ON-Time and conventional sequencing and a 100% agreement in samples where *Enterobacteriaceae* is the most abundant family (Figures 3D–3F). Overall agreement between sequencing methods for other common families, *Bacteroidaceae* abundance and *Prevotellaceae* abundance, was 94% (Figure 3F). Therefore, these data confirm that ON-Time rapid microbiome analysis provides robust data when compared to the current "gold-standard" of short-read shotgun sequencing.

Functional genomics analysis of AMR and virulence determinants using ON-Time

In addition to microbiome community and taxonomic metrics, other potentially clinically actionable data from microbiome analysis includes functional genomics such as antimicrobial

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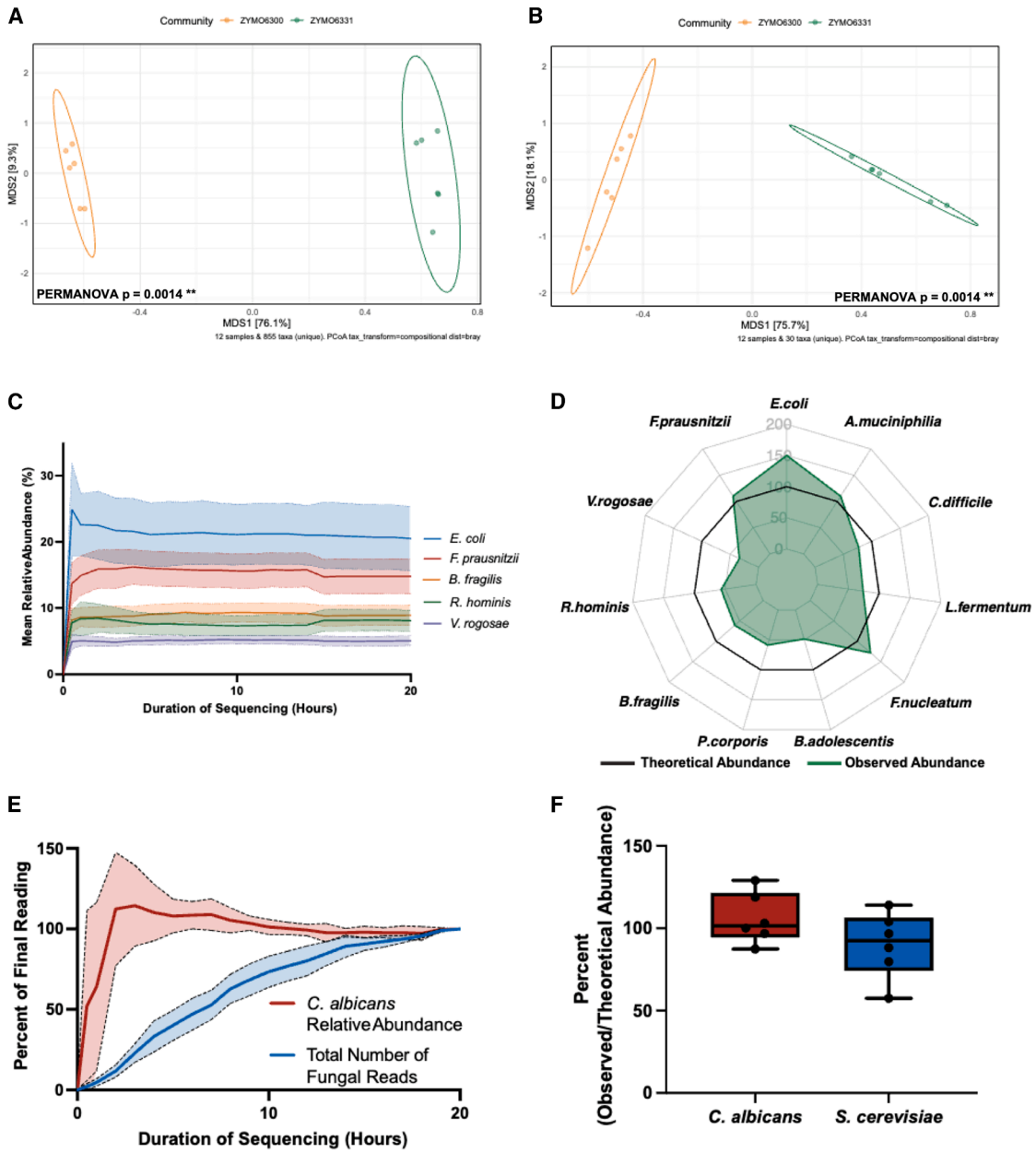

Figure 2. Validation ON-Time rapid microbiome analysis using standardized microbial communities

(A) Multidimensional scaling (MDS) plot (Bray-Curtis dissimilarity distances, species level) of ON-Time analysis of bacterial communities in ZymoBIOMICS D6300 standard (yellow, $n = 6$ independent replicates) and the ZymoBIOMICS D6331 standard (green, $n = 6$ independent replicates). p value = 0.0014 by PERMANOVA.

(B) Multidimensional scaling (MDS) plot (Bray-Curtis dissimilarity distances, species level) of ON-Time analysis of fungal communities in ZymoBIOMICS D6300 standard (yellow, $n = 6$ independent replicates) and the ZymoBIOMICS D6331 standard (green, $n = 6$ independent replicates). p value = 0.0014 by PERMANOVA.

(C and D) Real-time analysis of relative abundance values of five bacterial species in the ZymoBIOMICS D6331 standard using ON-Time sequencing. Data are reported as percentage of final reading (i.e., reading at 20 h of sequencing) at each time point, line shows mean, and shaded area shows standard deviation ($n = 6$ independent replicates) (D) Radar plot of the observed to theoretical relative abundance (expressed as percentage) of 11 bacterial species in the ZymoBIOMICS D6331 standard using ON-Time analysis ($n = 6$ independent replicates).

(E) Real-time analysis of fungal (*Candida albicans*) relative abundance as well as total number of fungal reads in the ZymoBIOMICS D6331 standard using ON-Time sequencing. Data are reported as percentage of final reading (i.e., reading at 20 h of sequencing) at each time point, line shows mean, and shaded area shows standard deviation ($n = 6$ independent replicates).

(F) Plot of the observed to theoretical relative abundance (expressed as percentage) of two fungal species in the ZymoBIOMICS D6331 standard using ON-Time ($n = 6$ independent replicates; line shows median, box shows IQR, and whiskers show range).

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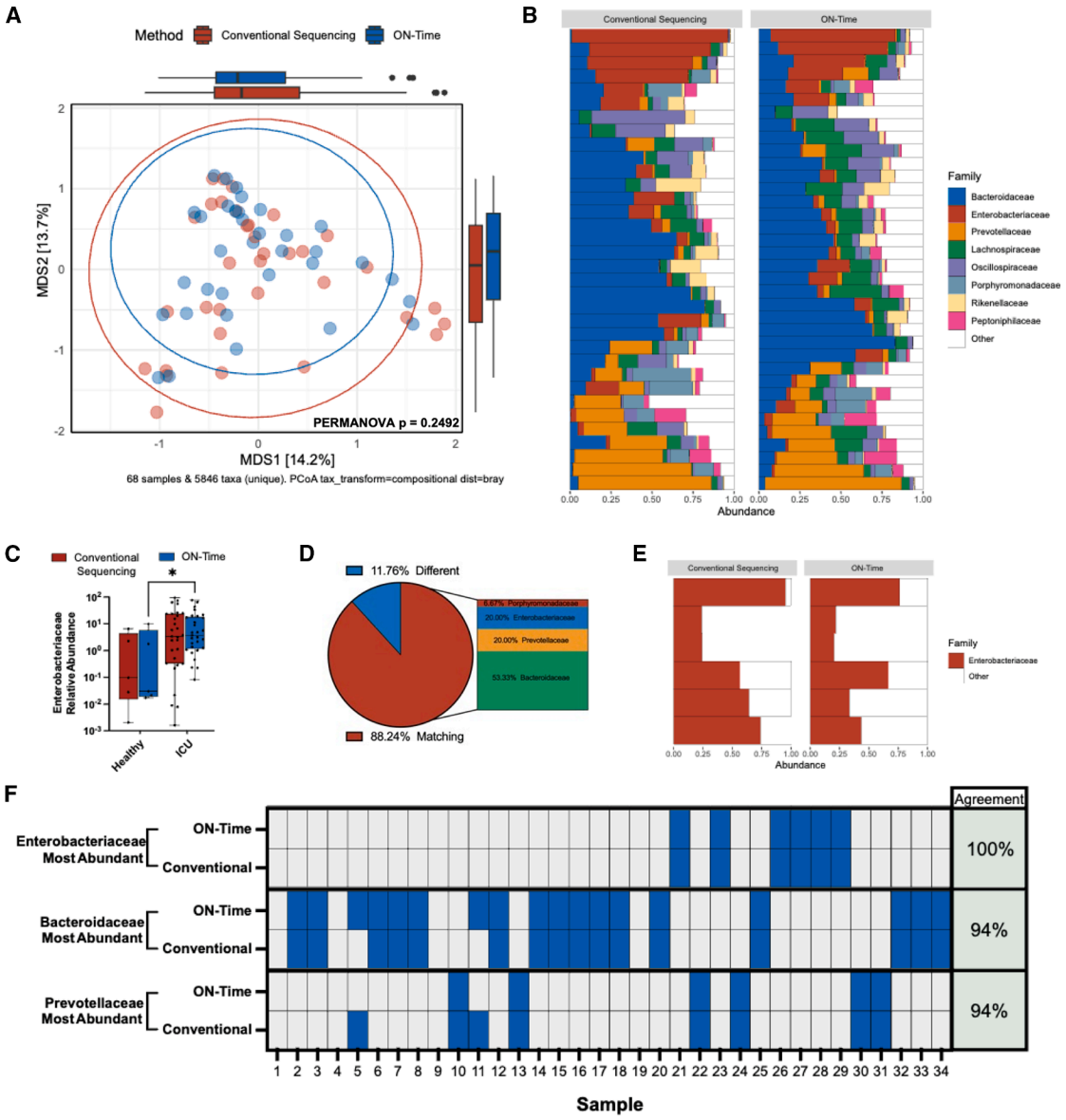

Figure 3. Comparison of microbiome data outputs between ON-Time and conventional shotgun metagenomics

Rectal swab samples were collected from healthy donors (n = 5) and ICU patients (n = 29), DNA was extracted, and then aliquots of the DNA underwent library preparation and sequencing using ON-Time as well as shotgun metagenomic sequencing on Illumina NovoSeq 6000.

(A) Multidimensional scaling (MDS) plot (Bray-Curtis dissimilarity distances, species level) of ON-Time analysis of bacterial communities in rectal swab samples analyzed by ON-Time (blue) versus conventional shotgun sequencing (red). p value = 0.2492 by PERMANOVA.

(B) Taxonomic composition (by relative abundance) of bacterial families in matched samples analyzed by ON-Time versus conventional shotgun sequencing.

(C) Enterobacteriaceae relative abundance in rectal swab samples from healthy (n = 5) and ICU patients (n = 29) sample determined by ON-Time versus conventional shotgun sequencing. Dots represent individual patients, line shows median, box shows interquartile range (IQR), and whiskers show range. Analyzed by a two-tailed Mann-Whitney U test (healthy control versus ICU within each method respectively). *$p < 0.05$.

(D) The percentage of healthy (n = 5) and ICU patient samples (n = 29) for which ON-Time and shotgun sequencing were concordant (matching) or discordant (different) in bacterial families identified as the most abundant in each patient sample. The callout is the percentage of the most abundant bacterial family in the sample when the most abundant family is the same between conventional sequencing and ON-Time.

(E) Relative abundance of *Enterobacteriaceae* (versus other taxa) reported by ON-Time versus shotgun sequencing in six ICU patient samples where *Enterobacteriaceae* was identified as the most abundance family.

(F) A heatmap representing microbiome metrics that are present (blue) or absent (gray) in each patient sample as determined by ON-Time or conventional shotgun sequencing. Agreement value is the percentage of samples in which the indicated metric was concordant between ON-Time and conventional shotgun sequencing.

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resistance gene (AMR) and virulence gene presence within metagenomes (or even metagenome-aligned genomes [MAGs]). To determine whether our ON-Time rapid microbiome analysis pipeline could be used for functional genomic interrogation of ICU patient fecal samples, we identified AMR and virulence genes within the metagenomes of ICU patient samples (Figures 4A and 4B). Running ICU patient samples individually through ON-Time, we quantified the counts of AMR and virulence genes in real time over the course of 20 h of sequencing (Figures 4C and 4D). Unique AMR and virulence genes were detected within 3 h of sequencing (Figures 4C and 4D); however, additional unique AMR and virulence genes, as well as additional copies of identified genes, continued to accumulate as sequencing progressed, with no apparent plateau during 20 h of sequencing (Figure 4C). The same kinetics were observed with virulence genes, with unique genes and additional copies being detected throughout the entirety of the sequencing run (Figure 4D). Therefore, despite excellent performance for taxonomic and community analyses, ON-Time rapid microbiome pipeline consisting of 3 h of sequencing time (or even 20 h of extended sequencing) demonstrated a limited ability to rapidly and comprehensively report AMR and virulence genes within ICU patient fecal samples.

DISCUSSION

In this study, we describe and validate a method to rapidly analyze microbiomes of patient fecal samples, called ON-Time. Our data demonstrate that ON-Time yields accurate and precise microbiome data from fecal (rectal swab) samples of ICU patients in <4.8 h. ON-Time results were validated using mock communities as well as head-to-head comparison with conventional short-read shotgun metagenomics. In contrast to conventional microbiome sequencing methods (16S rRNA gene amplicon sequencing and shotgun metagenomics), ON-Time analysis is cost-effective for on-demand individual patient sample analysis, simplified (fewer and simpler wet lab steps and point-and-click bioinformatics), and requires minimal infrastructure (sequencing and analysis accomplished with a laptop attached to Mk1B device). Taken together, ON-Time offers rapid, accurate, and cost-effective method to analyze individual patient's gut microbiome to identify actionable microbiome data for targeted (personalized) therapy.

The complexity and inter-individual heterogeneity of the gut microbiome necessitates individualization of microbiome modifying interventions. This is exemplified by large randomized controlled trials of microbiome-modifying treatments in critical care such as selective digestive decontamination as well as probiotics, where a one-size-fits-all approach to microbiome modification across diverse populations of patients did not result in improved clinical outcomes.[16,17] The ability to identify patients with appropriate microbiome characteristics for targeted interventions—such as identifying patients with *Enterobacteriaceae* overgrowth for digestive decontamination, patients with reduced commensal anaerobes for probiotic supplementation, or patients with other disease-associated dysbiosis features for targeted treatment (e.g., microbial consortia) is clinically valuable. Collectively, microbiome-guided approaches to treatments may help enrich clinical trials for treatment-appropriate subjects, thereby improving their power to detect treatment effects, as well as guide precision/personalized interventions in clinical practice (an example of a clinical workflow utilizing ON-Time for microbiome-guided care in ICU shown in Figure S3). Our data support the feasibility of using ON-Time analysis within the critical care setting to identify patients with treatable microbiome targets (pathobiont abundance and community biodiversity) that can be accomplished within the rapid timeline required for clinical decision-making in the ICU. As such, ON-Time is poised to help overcome key limitations of prior microbiome-modifying therapy trials in critical care by enabling precision application of interventions to the right patient at the right time.

Data presented in this study confirms that ON-Time overcomes critical limitations of conventional microbiome analysis methods for use in acute medicine, while yielding equivalent ecological data about patient sample microbiomes. First and foremost, the extended time required for 16S amplicon or shotgun metagenomic sequencing and analysis are unsuitable for time-critical clinical decision-making in the ICU and other acute medical settings. Even existing "rapid" metagenomic next-generation sequencing (mNGS) and targeted next-generation sequencing (tNGS) methods, which primarily utilize short-read sequencing of PCR-amplicon or capture-based DNA sequences, typically yield turnaround times of 24 h or more. Furthermore, the primary application of existing rapid microbial sequencing tools is infection diagnostics optimized for individual (or limited panel of) pathogens or gene targets, rather than complex microbiome metagenomics. ON-Time overcomes these limitation, yielding a clinically feasible turnaround time (<5 h) that can enable microbiome-driven care in the acute medical setting of the ICU. Furthermore, the relatively low taxonomic resolution of 16S amplicon sequencing produces microbiome profiles restricted to the genus (or higher) phylogenetic level, which would be insufficient for microbiome interventions that require knowledge of species- or strain-level composition. Furthermore, 16s amplicon sequencing provides information only on bacterial communities, which would necessitate additional methods to characterize other kingdoms (e.g., ITS amplicon sequencing for fungi). In contrast, ON-Time was found to yield accurate and detailed taxonomic data to species and even strain resolution of both bacterial and fungal communities. ON-Time was designed with real-world considerations for practicality and feasibility. ON-Time enables analysis of individual patient samples on demand at a relatively low cost, compared to conventional sequencing methods that are optimized for batched multiplexing of hundreds of samples, which makes on-demand individual patient analysis impractical and prohibitively expensive for clinical adoption. Lastly, we included a simplified point-and-click data analysis pipeline for FASTQ files to democratize the accessibility of ON-Time for non-experts in bioinformatics, thereby enhancing the practicality and feasibility for use in the real-world clinical setting.

Rapid microbiome analysis capability offered by ON-Time may be applicable to uses beyond gut microbiome profiling and beyond the critical care setting. Beyond the ICU, microbiome-directed care is being investigated in many other acute medical patient populations, including bone marrow transplant

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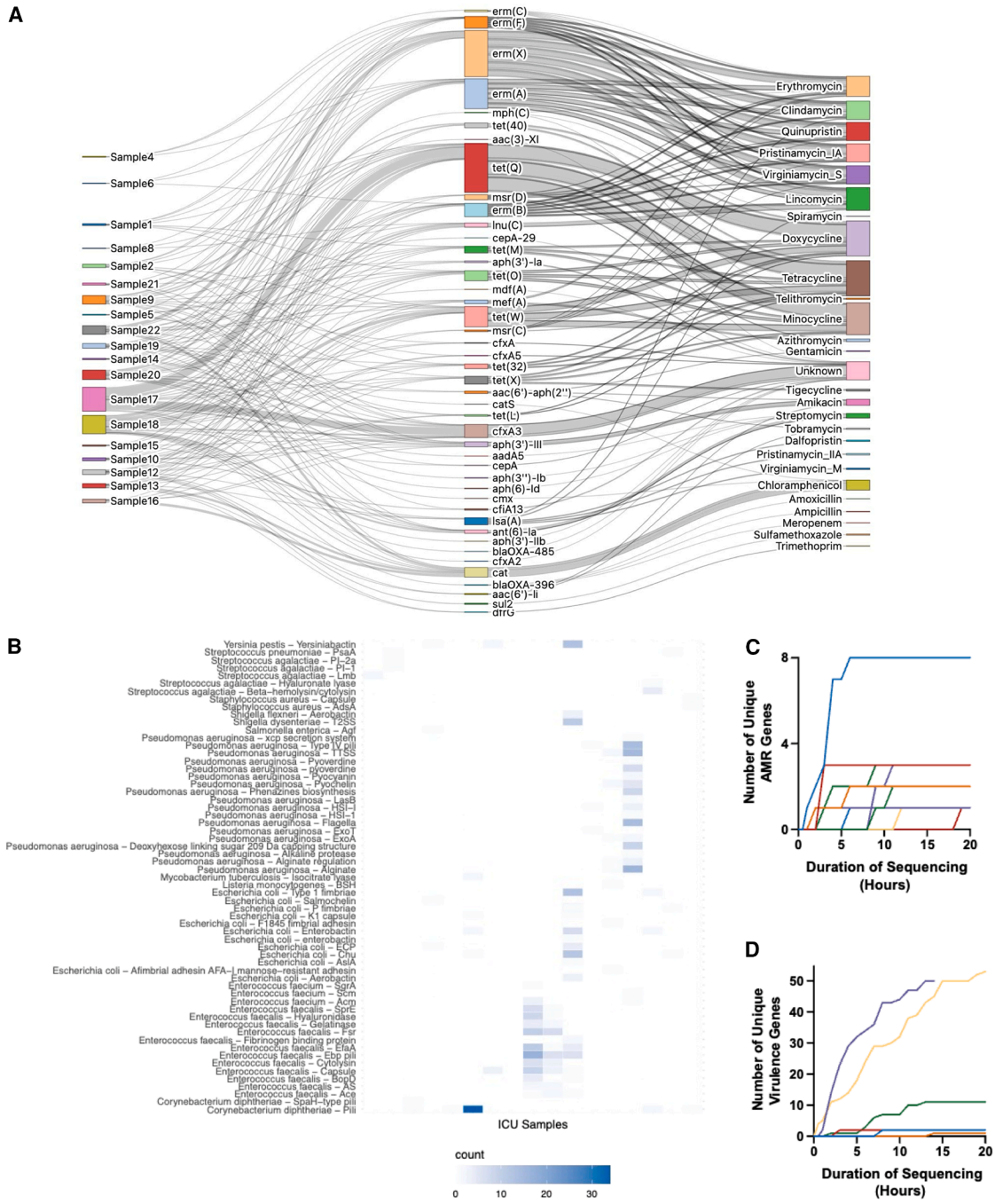

Figure 4. Functional genomic analysis of AMR and virulence genes in ICU patient samples using ON-Time

(A) Sankey diagram linking individual patient samples (left) to antimicrobial resistance genes identified and their corresponding antibiotic target.

(B) Heatmap of bacterial virulence factor genes identified in ICU patient rectal swab samples.

(C) Real-time analysis of the number of unique AMR genes detected in individual ICU patient samples (n = 19) and mock community samples (n = 12) using ON-Time analysis (eight patient samples and three mock community samples contained detected AMR genes; each color denotes a unique sample).

(D) Real-time analysis of the number of unique virulence genes detected in individual ICU patient samples (n = 19) and mock community samples (n = 12) using ON-Time analysis (three patient samples and three mock community samples contained detected virulence genes; each color denotes a unique sample).

patients, oncology, infectious diseases, and many others.[9] Although developed using ICU patient fecal samples in this study, the process of ON-Time microbiome analysis is disease agnostic and can be applied to any patient population. Importantly, as there are no universal metrics of microbiome dysbiosis, future studies as well as clinical utilization of ON-Time will require

identification and validation of disease-specific, outcomes-associated microbiome metrics and thresholds for each application. Furthermore, beyond our focus on the gut microbiome in this study, we propose that ON-Time may be used to analyze microbial composition of other body sides and sample types. However, inherent issues with other sample types such as sputum, urine, and skin, including low microbial biomass and/or high host DNA contamination, would require refinements to both wet- and dry-lab aspects of the ON-Time protocols. Lastly, ON-Time may be useful for additional clinical applications beyond identifying patients for microbiome-modifying therapies, including monitoring of treatment responsiveness (e.g., monitoring engraftment after fecal microbiota transplant therapy[18]), prognostication,[19–21] and even infection diagnostics. The unbiased nature of ON-Time sequencing may overcome limitations of current targeted microbiology diagnostic methods that requires *a priori* knowledge of the possible pathogens in a sample (such as culture-based or multiplexed PCR diagnostic testing). Thus, future research is warranted to investigate the multiple potential applications of ON-Time rapid microbiome analysis.

In summary, this study describes the development and validation of a rapid, cost-effective, and clinically feasible method for microbiome characterization. The goal of ON-Time rapid microbiome analysis is to enable timely, personalized microbiome-guided care in the ICU, which will be tested in forthcoming randomized controlled trials of precision microbiome-modifying therapies for critically ill patients.

Limitations of the study

ON-Time rapid microbiome analysis has several limitations. First, our data reveal that ON-Time analysis consisting of 3 h of sequencing run time is insufficient to conduct comprehensive interrogation of functional metagenomics, such as the confident identification and quantification of AMR or virulence genes in fecal microbiota. This may relate to the stochastic nature of identifying specific genes within microbiome samples as sequencing progresses, such that comprehensive metagenome coverage is the only way to confidently analyze functional microbiome genomics. Future research will be required to delineate the limits of sensitivity and specificity for AMR and virulence gene analysis (or other functional genomics) using ON-Time and whether the time-resolution of AMR and virulence gene detection can be improved via changes in sequencing chemistry or modality. Second, the parameters of ON-Time analysis in this study have been optimized for use with relatively high microbial biomass (and low host DNA) in rectal swab samples. As noted above, application of this method to other clinically important sample types, such as sputum that has much lower microbial biomass that is vastly outnumbered by host DNA, will likely require additional wet-lab pre-processing (e.g., host DNA depletion step), longer sequencing time (to accumulate sufficient microbial reads), and/or computational optimization (e.g., reference database optimization) to achieve high-quality data outputs. Lastly, our methodological development of ON-Time is based on published metrics for Shannon diversity index threshold and the relative abundance of gut pathobionts in ICU patients.[3] However, as larger datasets from diverse patient cohorts become available, these thresholds should be refined and stratified according to patient demographics. Additionally, future efforts must be directed toward a more comprehensive enumeration of these gut pathobionts. While our study focused on pathobiont taxa with moderate to high abundance, the performance of ON-Time for detection and relative quantification of rare and low-abundance taxa, as well as descriptive microbial community metrics were not assessed in detail and may require additional validation against conventional short-read methods and/or adjustments to the sequencing time to optimize performance of ON-Time for these metrics. Lastly, forthcoming prospective clinical trials are required to assess the feasibility and impact of ON-Time toward microbiome-guided care in the clinical arena (clinicaltrials.gov NCT07463339).

RESOURCE AVAILABILITY

Lead contact

Requests for further information or resources should be directed to the lead contact, Dr. Braedon McDonald (bamcdona@ucalgary.ca).

Materials availability

This study did not generate unique reagents.

Data and code availability

- Sequencing data presented in this paper are all publicly available in the NCBI BioProject ID: PRJNA1433842.
- The analysis pipeline utilizes publicly available software referenced in the STAR Methods section and key resources table. We have also developed a web app for implementation: https://apps.thebiohub.ca/ONTime/, and the script has also been deposited at Zenodo with doi:10.5281/zenodo.21811306
- Any additional information required to re-analyze the data reported by this study is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

B.McD., C.S.T., and K.D.McC. conceived the study, designed experiments, secured funding, and provided overall supervision. I.B. and B.McD. enrolled participants, and I.-L.Y., I.B., B.McD., and J.S. collected, processed, and stored patient samples. C.M. designed and performed experiments. C.M., J.S., A.H., and B.McD. analyzed the data and prepared figures. C.M., H.S., and B.McD. prepared the manuscript. All authors edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
ZymoBIOMICS D6300 Microbial Community Standard	Zymo Research	Cat# D6300
ZymoBIOMICS D6331 Gut Microbiome Standard	Zymo Research	Cat# D6331
Biological samples		
Human rectal swab microbiome samples	This paper and Schlechte et al.[3]	REB18-1294
Critical commercial assays		
QIAGEN DNeasy PowerSoil Kit	QIAGEN	Cat# 47016
ONT Native Barcoding Kit 96 V14	Oxford Nanopore Technologies	Cat# SQK-NBD114.96
ONT Rapid Barcoding Kit 24 V14	Oxford Nanopore Technologies	Cat# SQK-RBK114.24
Deposited data		
Raw FASTQ Metagenomic Sequencing Data	This paper	NCBI BioProject ID PRJNA1433842
Software and algorithms		
ON-Time web-based application	This paper	https://apps.thebiohub.ca/ONTime/
FastQC (v0.11.8)	Andrews[22]	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
Sickle (v1.33)	Joshi & Fass[23]	https://github.com/najoshi/sickle
MinKNOW	Oxford Nanopore Technologies	https://nanoporetech.com/software/devices/minion-mk1b
BugSeq	BugSeq	https://bugseq.com
Epi2Me wf-metagenomics (v.2.11.0)	Oxford Nanopore Technologies	https://github.com/epi2me-labs/wf-metagenomics
Kraken2 (v.2.1.3)	Wood et al.[24]	https://doi.org/10.1186/s13059-019-1891-0
ShortRead (v1.68.0)	Morgan et al.[25]	https://doi.org/10.1093/bioinformatics/btp450
kraken-biom (v1.0.1)	Dabdoub et al.[26]	https://github.com/smdabdoub/kraken-biom
phyloseq (v1.50.0)	McMurdie & Holmes[27]	https://doi.org/10.1371/journal.pone.0061217
decontam (v.1.26.0)	Davis et al.[28]	https://doi.org/10.1186/s40168-018-0605-2
microViz (v0.12.5)	Barnett et al.[29]	https://doi.org/10.21105/joss.03201
ggplot2 (v3.5.1)	Wickham, H.[30]	https://doi.org/10.1007/978-3-319-24277-4.
microbiome (v1.28.0)	Lahti et al.[31]	https://github.com/microbiome/microbiome
dplyr (v1.1.4)	Wickham et al.[32]	https://github.com/tidyverse/dplyr
GraphPad Prism (v10.4.1)	Dotmatics	https://www.graphpad.com
vegan (v2.7-3)	Oksanen et al.[33]	https://cran.r-project.org/web/packages/vegan/index.html

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Study design and participants

This study was approved by the Conjoint Health Research Ethics Board (CHREB) of the University of Calgary and Alberta Health Services (REB18-1294). The study outline of sample collection, inclusion criteria, and exclusion criteria have been previously reported.[3] The age range of patient samples analyzed in this study is 20–86 years old. For this study in brief, rectal swabs samples were collected from patients admitted to the medical, surgical, neurological, and trauma ICUs at the Foothills Medical Center in Calgary. The enrollment dates for the clinical samples described herein occurred between 23 July 2019 and 27 May 2025.

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METHOD DETAILS

DNA isolation from ICU rectal swabs, mock communities, and controls

The DNA isolation from rectal swabs was performed as previously described.[3] Briefly, rectal swabs stored in sterile tubes at −80°C were thawed at 4°C, then shaken at 30Hz for 1-min in 1mL of phosphate buffered saline (PBS), pelleted, and the supernatant was removed from the sample. The DNA from the pellet was then isolated using the DNeasy PowerSoil Kit (QIAGEN), following the manufacturers protocol, with the nuclease-free water as a negative control extracted with every sample run. In addition to ICU fecal swab samples, DNA was extracted following the same protocol from both the ZymoBIOMICS D6300 Microbial Community Standard and the ZymoBIOMICS D6331 Gut Microbiome Standard (Zymo Research). Following isolation, dsDNA was quantified using a Nanodrop One Spectrophotometer (Thermo Scientific).

Oxford Nanopore Technologies library preparation and sequencing

For the comparison study between Illumina and ONT sequencing, isolated DNA from fecal swabs, negative controls, and the ZymoBIOMICS mock communities underwent library preparation using the ONT Ligation sequencing gDNA – Native Barcoding Kit 96 V14 (SQK-NBD114.96), with protocol version NBE_9171_v114_revQ_23Dec2024 according to the manufacturer's instructions (Oxford Nanopore Technologies). The samples were run using the MinKNOW software[34] on a ONT PromethION flow cell for 120-h, with super accurate basecalling. For the rapid ON-Time sequencing pipeline, isolated DNA from fecal swabs, negative controls, and the ZymoBIOMICS mock communities underwent library preparation using the ONT Rapid Barcoding Kit SQK-RBK114.24, with protocol version RBK_9176_v114_revM_27Nov2022 according to the manufacturer's instructions (Oxford Nanopore Technologies). The individual ICU DNA sample and the negative control were run on an ONT Flongle flow cell for up to 24-h, with super accurate basecalling. For the multiplexed rapid ICU samples, negative controls, and the ZymoBIOMICS mock community underwent library preparation using the same SQK-RBK114 protocol as the individual samples but were run on an ONT MinION flow cell for 72-h. Following each ONT sequencing run, FASTQ files are generated post-basecalling and are available for downstream analysis.

Illumina shotgun metagenomic sequencing

Conventional shotgun metagenomic sequencing was completed at the International Microbiome Center (Calgary, AB) using an Illumina NovaSeq 6000 (Illumina) to produce paired end reads. Initial quality control of the raw sequencing was completed using the FastQC software (version 0.11.8) in command-line.[22] Following quality control confirmation, raw forward and reverse read FASTQ files are trimmed using the Sickle software (v1.33) in command-line.[23] Following trimming of the shotgun metagenomic reads, forward and reverse read cleaned-FASTQ files are available for downstream analysis.

QUANTIFICATION AND STATISTICAL ANALYSIS

Metagenomic DNA data processing and statistical analysis

For the comparison study between Illumina and ONT sequencing, raw sequencing output files were processed using BugSeq and the BugRef Curated DB (BugSeq, Vancouver).[35] When analyzing individual ICU samples run on the ONT Flongle flow cell, the analysis was possible through usage of the ONT Epi2Me wf-metagenomics workflow (v.2.11.0), using the curated Kraken2 databases: HumGut[36] for prokaryotes and PlusPF-8 for fungal analysis (Oxford Nanopore Technologies). Within the Epi2Me wf-metagenomics (v.2.11.0), all default settings are used. Kraken2 (v.2.1.3) is used with the default parameters including: taxonomic_rank = S, confidence score = 0.0, and disabled memory mapping; in addition the wf-metagenomics minimum read length remains the default 0. The AMR and virulence analysis utilized the same Epi2Me wf-metagenomics (v.2.11.0), with the addition of a built-in Abricate AMR search using the built-in Resfinder database, and the Abricate virulence search using built-in VFDB (Oxford Nanopore Technologies) with the default Abricate settings: AMR identity threshold = 80, and AMR coverage = 80. Real-time analysis of the samples is made possible through continuous monitoring of the export FASTQ files by the Epi2Me software however, real-time analysis was also achievable post-hoc with the export and subsequent binning of FASTQ files in intervals throughout the sequencing run. The read length and read count information from each generated FASTQ file is determined using the R ShortRead package (v1.68.0).[25] Following all metagenomic searches above, the files are available in a Kraken-report format, from which the results are simplified into a BIOM table format using the command-line tool kraken-biom (v1.0.1).[26] The BIOM table format is then imported into R for downstream analysis. The BIOM table format is combined with sample data using the R phyloseq package (v1.50.0),[27] contaminants are then removed using the respective negative control sample for each sequencing run using the R decontam package v.1.26.0,[28] followed by removal of cyanobacteria and mitochondria contaminants from the classification. Host DNA contamination (*Homo sapiens*) is removed following the creation of the phyloseq object through the deletion of any data from taxa that do not belong to kingdoms Fungi or Bacteria. Relative abundance plots are created using the R package microViz (v0.12.5).[29] The principal component analysis was calculated in the microViz,[29] and ellipses are added using the R ggplot2 package (v3.5.1).[30] The community dissimilarity (β-diversity) was calculated on the Bray-Curtis dissimilarity measure by PERMANOVA using the distance function in microViz.[29] Community α-diversity metrics including Shannon diversity index and Chao1 were calculated using the R microbiome package (v1.28.0),[31] which also generates the number of overserved taxa in each sample.

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The calculation of the elbow of the curve for both Shannon diversity index and *Enterobacteriaceae* relative abundance according to time was calculated using a custom R script utilizing the Kneedle Method.[37] Briefly, the time and relative abundance values are normalized, a unit vector is drawn from the minimum-maximum normalized value and the furthest corresponding point to this vector represents the elbow of the curve.[37] The corresponding time value belonging to the elbow of the curve is determined using the R dplyr package (v1.1.4).[32] The total number of bacterial reads were found using the phyloseq and the R dplyr package (v1.1.4).[27,32] Relative abundance differences between methods were analyzed using a two-tailed Mann-Whitney U-test (healthy control versus ICU within each method respectively) in GraphPad Prism (v10.4.1). Concordance of sequencing methods was calculated by first finding the compositional Bray-Curtis dissimilarities for each method individually at the species and family level using base R statscdmscale(), followed by matching samples across each sequencing method then computing the Procrustes values using the function veganprocrustes() with the significance of the results determined by Protest analysis with $n = 9999$ permutations using the function veganprotest() in the R vegan package (v2.7-3).[33]